

## Embryonic and larval development of *Pinctada margaritifera* (Linnaeus, 1758)

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### Abstract

We describe the developmental stages of the black-lip pearl oyster, *Pinctada margaritifera* (Linnaeus, 1758), larvae from fertilisation through embryonic development and larval growth in the laboratory at  $28 \pm 1^\circ\text{C}$ . Larvae were anaesthetised, fixed, critical-point dried and examined using a scanning electron microscope. We examined embryonic development (fertilisation, polar body, blastomeres, gastrula) and attributes of the larval shell (size, prodissoconch I/II, growth lines, provinculum, shell fracture) and larval velum. The first polar body formed 24 min after fertilisation and fertilised eggs had a mean diameter of  $59.9 \pm 1.4 \mu\text{m}$ . The earliest actively swimming trochophore appeared 8–12 h after fertilisation. The D stage was reached approximately 24 h after fertilisation and measured  $79.7 \pm 2.3 \mu\text{m}$  in shell length. Ten-day-old larvae had umbones that arose opposite each other above the hinge axis and 22-day-old larvae, with a mean shell length of  $230.8 \pm 4.9 \mu\text{m}$ , developed a pigment spot just before entering the pediveliger stage.

*Additional keywords:* hinge, morphology, pearl oyster, scanning electron microscope.

### Introduction

Herdman (1903) studied the early life stages of *Pinctada vulgaris* (= *P. fucata* Gould, 1850) larvae up to 3 days after fertilisation. Other studies have investigated larval development and growth of *P. fucata*, *P. martensi* Dunker, 1850 and *P. maxima* Jameson, 1901 (Ota 1957; Minaur 1969; Tanaka and Kumeta 1981; Alagarwami *et al.* 1983). Rose and Baker (1994) described larval development of *P. maxima* in detail and compared their findings with those reported for other pearl oyster species. Although *P. margaritifera* larvae have been cultured since 1970 (Tanaka *et al.* 1970; Alagarwami *et al.* 1989; Southgate and Beer 1997), embryonic development and the morphology of different larval stages have not been described in detail.

The present study notes the characteristics of *P. margaritifera* larvae that have not been reported on previously using scanning electron microscopy (SEM) and provides a basic understanding of larval development during hatchery culture.

### Materials and methods

#### Larval rearing

*Pinctada margaritifera* broodstock were induced to spawn by thermal stimulation and the addition of sperm in a seawater suspension. Fertilised eggs were stocked at a density of  $30 \text{ mL}^{-1}$  in aerated fibreglass tanks (500 L) filled with  $1 \mu\text{m}$  filtered seawater at  $28^\circ\text{C}$ . The salinity of seawater was 33, which was measured using the practical salinity scale. After 24 h, when D-stage larvae (shell becomes D-shaped) had a mean shell length of  $79.7 \pm 2.3 \mu\text{m}$ , they were collected on a  $25\text{-}\mu\text{m}$  mesh sieve, counted and placed at a density of  $2 \text{ mL}^{-1}$  in 500-L aerated fibreglass tanks containing filtered seawater at the same temperature and salinity.

We cultured Tahitian *Isochrysis* aff. *galbana* Green (T-ISO) and *Pavlova salina* Green in 3-L glass flasks and 20-L carboys in autoclaved  $0.45\text{-}\mu\text{m}$ -filtered and ultraviolet (UV)-treated seawater using f/2 nutrient



medium (Guillard 1983). Microalgae cultures were provided with illumination from cool white fluorescent lights with a 12-h light:12-h dark photoperiod. Larvae were fed daily a 1:1 mixture of T-ISO and *Pavlova salina* at a ration of  $1-18 \times 10^3$  cells  $\text{mL}^{-1}$  (Southgate and Beer 1997; Doroudi *et al.* 1999a). We conducted three separate spawnings to collect embryonic and larval samples.

#### *Sample preparation*

We observed embryonic development every 15 min during the first 3 h, then once an hour until the trochophore stage (8–24 h) and D-stage (24 h) using a compound microscope. Larvae were narcotised in a 15% (w/v) solution of  $\text{MgCl}_2$  (Bellolio *et al.* 1993) and seawater (1:1) at 28°C for 5–10 min. We collected samples from three tanks at 2-day intervals after the D stage (24 h) and fixed them in 2.5% glutaraldehyde in 0.1 M piperazine at pH 7.6. This sampling continued until larvae had developed to the 'eyed' stage (i.e. when larvae develop a pigment spot). Subsamples of larvae were post-fixed with osmium tetroxide ( $\text{OsO}_4$ ), dehydrated in a graded series of ethanol, critical-point dried in liquid carbon dioxide ( $\text{CO}_2$ ), mounted on aluminium stubs with double-sided tape and coated with gold before being examined using an SEM.

We collected larvae on a mesh sieve, washed them into a graduated cylinder and removed a subsample, from which the shell length of 40 larvae was measured using a compound microscope. Morphological terminology follows that commonly used for bivalve larvae in similar studies (Waller 1981; Bellolio *et al.* 1993).

### **Results**

The time series of developmental stages of *P. margaritifera* embryos and larvae is shown in Table 1.

#### *Embryo to trochophore*

Unfertilised *P. margaritifera* eggs had a mean diameter of  $39.7 \pm 1.3 \mu\text{m}$  ( $n = 40$ ; Fig. 1). The first polar body formed 24 min after fertilisation and fertilised eggs had a mean diameter of  $59.9 \pm 1.4 \mu\text{m}$  ( $n = 40$ ). The four blastomeres resulting from the second cleavage formed 2 h after fertilisation. Cell division followed the usual bivalve pattern for bivalves and resulted in the gastrula, 5 h after fertilisation. The change from a ciliated gastrula to the trochophore stage was gradual and the earliest actively swimming trochophore appeared 8–12 h after fertilisation. Morphological changes from trochophore to the D stage included extension along the longitudinal axis and the apical region becoming broader than the posterior region. At this time, cilia on the apical region became longer. With the development of long cilia, larvae began to secrete shell and the resulting larvae swam actively using the velum.

#### *Larvae*

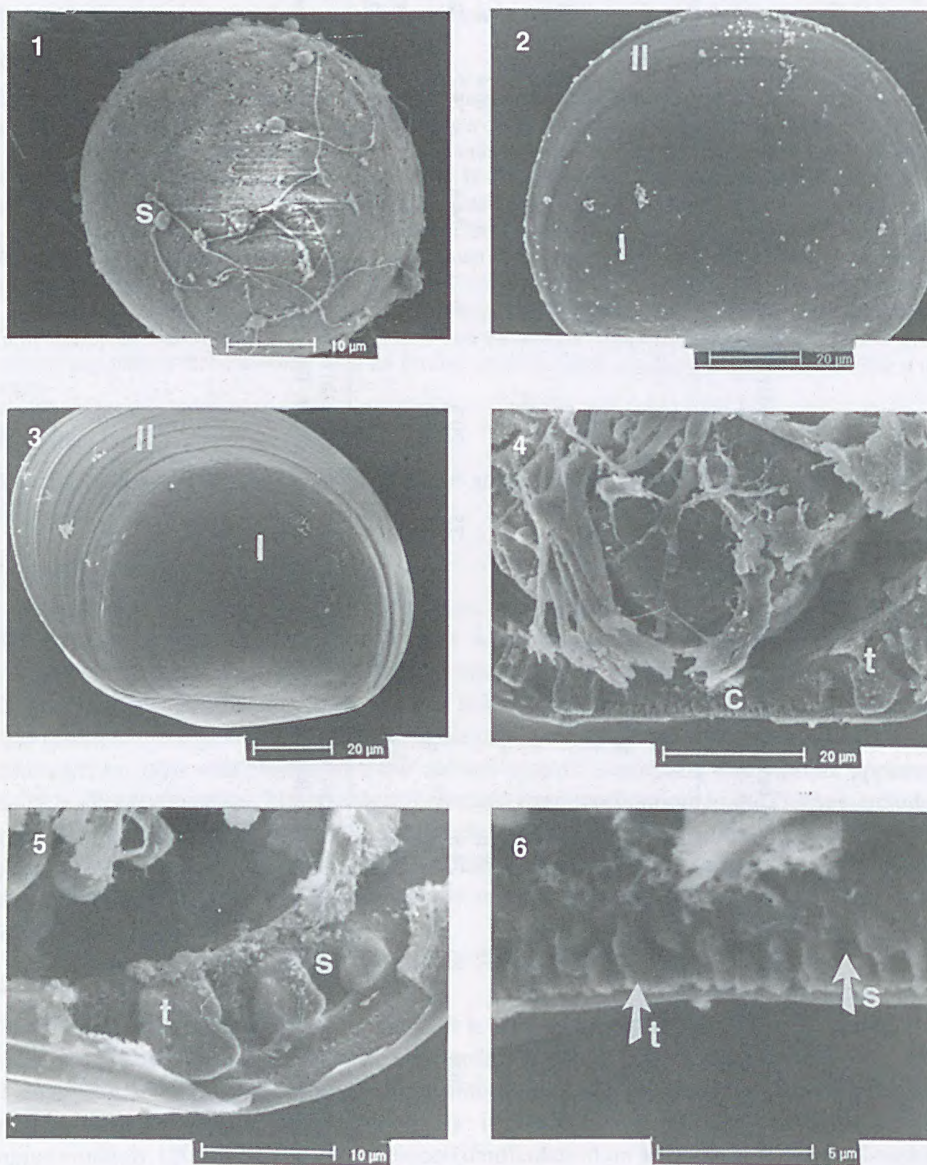
The D stage was reached approximately 24 h after fertilisation and larvae measured  $79.7 \pm 2.3 \mu\text{m}$  ( $n = 40$ ) in shell length. The D-stage larvae showed preliminary growth rings after 2 days (Fig. 2). The shell showed slight umbonal growth after 6 days development and prodissoconch I and II could be clearly identified (Fig. 3). At a shell length of approximately 100  $\mu\text{m}$ , the hinge developed denticulation on either side of a central region. As the larva grew, the hinge developed and formed a series of teeth and sockets on each valve (Fig. 4). Each valve had teeth on either side of a central area (Fig. 5). The central area included a series of tiny teeth and sockets (Fig. 6). Ten-day-old larvae had umbones that arose opposite each other above the hinge axis (Fig. 7) and 22-day-old larvae, with a mean shell length of  $230.8 \pm 4.9 \mu\text{m}$  ( $n = 40$ ), developed a pigment spot and entered the pediveliger stage shortly after (Fig. 8). Sections of broken shell edges at this stage suggest that calcification of the shell by the mantle had occurred in prodissoconch II (Fig. 9). Fractures through prodissoconch II showed layering in the shell structure and indicated that the shell is thicker in the area of growth lines (Fig. 10). The oval velum was located at the

Table 1. Embryo and larval development of some important pearl oyster species

| Authors*                      | Temperature (°C) | Stage     | Egg | Polar body | Four blastomere | Gastrula | Trochophore | D shape | Early umbo | Umbo  | Eye-spot |
|-------------------------------|------------------|-----------|-----|------------|-----------------|----------|-------------|---------|------------|-------|----------|
| <i>Pinctada fucata</i>        | 1                | 24.3–29.8 | S   | 47         | —               | —        | —           | —       | 100        | 135   | 210      |
|                               |                  |           | T   | —          | —               | —        | —           | 20 h    | —          | 11 d  | 15 d     |
|                               | 2                | 25.0–29.5 | S   | —          | —               | 60       | 75          | 85      | 110        | 140   | 235      |
|                               |                  |           | T   | —          | 1 h             | —        | 8 h         | 24 h    | 8 d        | 14 d  | 21 d     |
| <i>Pinctada maxima</i>        | 3                | 27–31     | S   | 59         | —               | —        | —           | —       | 96         | 125   | —        |
|                               |                  |           | T   | —          | —               | —        | —           | —       | 5 d        | 12 d  | —        |
|                               | 4                | 26–28     | S   | —          | —               | —        | —           | 75      | 115        | —     | —        |
|                               |                  |           | T   | —          | —               | —        | —           | 20 h    | 10 d       | —     | —        |
| <i>Pinctada margaritifera</i> | 5                | 26–30     | S   | 45         | —               | —        | —           | 75      | 110        | 140   | 210      |
|                               |                  |           | T   | —          | —               | —        | —           | 24 h    | 9 d        | 12 d  | 16 d     |
|                               | 6                | 27–29     | S   | 49.7       | —               | 60       | 69.6        | 79.7    | 109.9      | 140.7 | 230.8    |
|                               |                  |           | T   | —          | 24 min          | 2 h      | 5 h         | 8–12 h  | 24 h       | 8 d   | 22 d     |

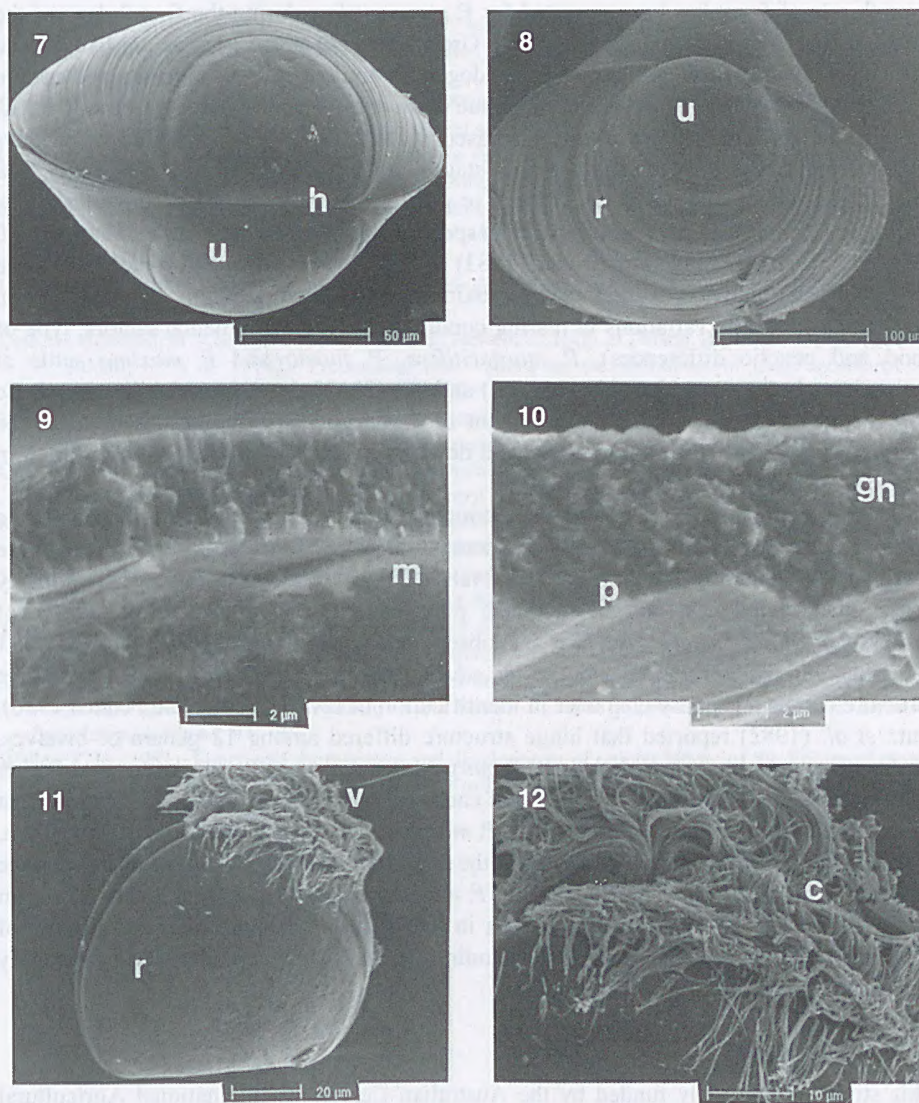
Mean size (S) of either the egg or larval diameter or shell length (in  $\mu\text{m}$ ); time (T) is given in minutes (min), hours (h) or days (d).  
 \*The sources of the data are as follows: 1, Alagarwami *et al.* (1983); 2, Rose and Baker (1994); 3, Minaur (1969); 4, Tanaka and Kumeta (1981); 5, Alagarwami *et al.* (1989); 6, present study.





**Figs 1–6.** 1, Unfertilised egg of *Pinctada margaritifera* with sperm (s) on the surface. 2, External view of the right valve of *Pinctada margaritifera* D-stage larvae; I, prodissococonch I; II, prodissococonch II. 3, Prodissococonch I (I) and II (II) in the early umbo stage of *Pinctada margaritifera* larvae. 4, Development of the provinculum of *Pinctada margaritifera* larvae (umbo stage); t, tooth; c, central area. 5, Main teeth on either side of a central area of *Pinctada margaritifera* larvae (umbo stage); t, tooth; s, socket. 6, Central area of the *Pinctada margaritifera* larval hinge (umbo stage) showing the series of teeth (t) and sockets (s).





**Fig. 7–12.** 7, Hinge length (h) and umbones (u) arise over the hinge axis of *Pinctada margaritifera* larvae (umbo stage). 8, An eyed larva of *Pinctada margaritifera* showing details of umbonal features (u); r, right valve. 9, The mantle (m) viewed from a cross-section of the prodissococonch II (II) of *Pinctada margaritifera* larvae (umbo stage). 10, Fracture of the prodissococonch in *Pinctada margaritifera* umbo larva; p, outer prismatic layer; gh, granular homogeneous layer. 11, Lateral view of the entire *Pinctada margaritifera* D-stage larva with velum (v) extended at the anterior dorsal side of the shell; r, right valve. 12, Enlargement of cilia (c) on the velum of *Pinctada margaritifera* D-stage larvae.

posterior dorsal side of the larva (Fig. 11) and was well developed with a peripheral ring of cilia (Fig. 12).

### Discussion

*Pinctada margaritifera* larvae need a period of 8 days to reach the early umbo stage (shell length 110  $\mu\text{m}$ ) and exhibit an average daily growth rate of 3.7  $\mu\text{m}$ . Elsewhere, a daily



growth rate of 5  $\mu\text{m}$  has been reported for *P. margaritifera* during the first 7 days of the larval rearing period (Tanaka *et al.* 1970). Growth rates of bivalve larvae are likely to be influenced by genetic factors, as well as endogenous and exogenous nutrition and culture conditions. Because *P. margaritifera* larvae have exponential growth (Doroudi *et al.* 1999b), the mean daily growth rate increased up to 7.2  $\mu\text{m}$  over the period of 22 days required for larvae to reach the eye spot stage (230  $\mu\text{m}$ ). Eye spots in *P. margaritifera* generally occur in larvae that are 230  $\mu\text{m}$  or greater in shell length. In a previous study, *P. margaritifera* larvae developed a pigment spot at 210  $\mu\text{m}$  shell length (Alagarwami *et al.* 1989). In *P. fucata* (Alagarwami *et al.* 1983) and *P. maxima* (Rose and Baker 1994), eye spots form in individuals that are approximately 210 and 230  $\mu\text{m}$  in shell length, respectively. Despite variations in rearing conditions (e.g. environmental factors, type of food and genetic differences), *P. margaritifera*, *P. fucata* and *P. maxima* settle at approximately the same size (230–266  $\mu\text{m}$ ) and age (20–23 days) from fertilisation (Rose and Baker 1994). The overall development of *P. margaritifera* larvae described in the present study is similar to the more general descriptions of pearl oyster larvae reported in previous studies (Table 1).

The larvae of bivalves are similar in exterior appearance and are difficult to differentiate without detailed anatomical study. The present SEM study of *P. margaritifera* larvae revealed some anatomical features of the larval shell that have not been observed previously using other techniques. For instance, the punctate region on the exterior surface of prodissoconch I of *P. margaritifera* is also observed in *Crassostrea virginica* Gmelin, 1791 (Carriker and Palmer 1979) and *Ostrea edulis* Linnaeus, 1750 (Waller 1981). Hinge structure can be a primary character in identification of bivalve larvae (Le Pennec 1980). Lutz *et al.* (1982) reported that hinge structure differed among 12 genera of bivalves, whereas the basic hinge morphology of *P. margaritifera* seems to be similar to that of other pearl oysters; that is, a tooth and socket at each end with a thin central area. The present study has shown that 8-day-old larvae of *P. margaritifera*, with a shell length of 110  $\mu\text{m}$ , have five teeth in each valve, with three at the anterior end of the hinge line and two at the posterior. This is the same as reported for *P. maxima* larvae with a shell length of 90  $\mu\text{m}$  (Rose and Baker 1994). Our observations in the present study on the larval rearing of *P. margaritifera* provide a basic understanding of larval development during hatchery culture of this species.

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